JACC Vol 2, No 4
October 1983:671–80

Role of Cellular Proteinases in Acute Myocardial Infarction

I. Proteolysis in Nonischemic and Ischemic Rat Myocardium and the Effects of Antipain, Leupeptin, Pepstatin and Chymostatin Administered in Vivo

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To test the hypothesis that cellular proteinases contribute to ischemic myocellular death, measurements were made of tyrosine release (an index of overall proteolysis) from incubated slices of nonischemic and ischemic myocardium obtained at various times after coronary artery occlusion in rats. Proteolysis failed to increase in ischemic myocardium throughout the first 24 hours of occlusion, when irreversible damage develops, indicating that cellular proteinases do not undergo generalized activation in this phase. These data represent the first assessment of myocardial proteolysis throughout the development of ischemic death, and suggest that cellular proteinases do not play a causal role in this process.

However, the possibility remains that ischemia selectively accelerates the breakdown of vital proteins, a phenomenon that may not be detected by measuring overall proteolysis. To determine whether future studies on the effects of proteolytic inhibition on infarct size are feasible, the ability of the proteinase inhibitors antipain, leupeptin, pepstatin and chymostatin, given in vivo, to interfere with proteolysis in ischemic myocardium was also evaluated. Leupeptin (10 or 40 mg/kg) inhibited proteolysis in a dose-related fashion (−49 and −72%, respectively, $p < 0.001$). Antipain (20 mg/kg) decreased protein breakdown by 60% ($p < 0.001$). The combination of antipain (20 mg/kg), leupeptin (40 mg/kg) and pepstatin (5 mg/kg) suppressed proteolysis almost completely at both 15 minutes (−88%, $p < 0.001$) and at 6 hours (−72%, $p < 0.05$) of ischemia, that is, throughout the development of irreversible injury.

These results demonstrate that whatever proteolysis is occurring during acute myocardial infarction is largely mediated by cathepsins A, B, D, L and H and by calcium-activated neutral protease (that is, the enzymes sensitive to the inhibitors used). Because antipain, leupeptin and pepstatin significantly suppress such proteolysis, these agents might be useful in further assessing any potential contribution of cellular proteinases to the production of ischemic myocellular death. In addition, this study provides a new experimental model that affords serial assessments of regional myocardial proteolysis during the evolution of myocardial infarction.

In recent years, considerable interest has focused on the possible role of lysosomal enzymes in the production of irreversible damage during acute myocardial infarction. Lysosomal enzymes undergo a subcellular redistribution and a shift from the particle-bound to the nonsedimentable fraction of tissue homogenates (1–10). Biochemical, immunohistochemical and ultrastructural studies have demonstrated that cathepsin D, a major lysosomal protease, is released from the lysosome and widely dispersed within the cytoplasm as early as 30 minutes after coronary artery occlusion (11–13), before the appearance of electron microscopic evidence of irreversible damage (9). These observations have led to the hypothesis that lysosomal proteinases contribute to irreversible damage by digesting vital cellular constituents early in the course of myocardial ischemia (11,14–16), as well as by leaking out of injured cells to attack adjacent, still viable myocytes (14).

This hypothesis is not supported by the finding that overall proteolysis decreases in isolated Krebs-Henseleit-perfused rat hearts during a short period (60 minutes) of flow reduction (17,18). However, data on proteolysis in the setting of *regional* acute myocardial ischemia produced in the in situ blood-perfused heart are lacking. Moreover, histochemical studies (19) suggest that in the rat model, my-

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ocellular damage in vivo progresses after the 60 minute period of ischemia explored in the previously cited experiments. Therefore, in the present investigation, we measured tyrosine production, an index of overall proteolysis (20), in nonischemic and ischemic myocardium of in situ blood-perfused rat hearts. These measurements were obtained serially over the entire first 24 hours of myocardial ischemia, during which time irreversible myocellular damage should have been completed.

The significance of proteolysis in ischemic myocardium might be further clarified by suppressing such activity in vivo and by evaluating the effect of this suppression on the magnitude of ischemic cell necrosis. Therefore, a second purpose of this investigation was to determine whether proteolysis could be inhibited by in vivo administration of several potent proteinase inhibitors recently developed by Umezawa et al. (21): leupeptin, an inhibitor of lysosomal cathepsins B, L, H (21) and of cytoplasmic calcium-activated neutral protease (22); antipain, an inhibitor of cathepsins A and B (21) and of calcium-activated neutral protease (22); pepstatin, an inhibitor of cathepsin D (21); and chymostatin, an inhibitor of cathepsins B and G (21). It was our plan to carry out a subsequent study on the effects of proteolytic inhibition on infarct size, if inhibition could be achieved.

Methods

Experimental preparation. Male Sprague-Dawley rats (230 to 270 g) were anesthetized with ether. A thoracotomy was performed in the left fifth intercostal space and the left coronary artery was ligated 2 to 4 mm below the level of the pulmonary valve as described previously (19). Fifteen minutes or 1, 2, 6, 18 or 24 hours after occlusion, the rats were stunned by a blow to the head and the heart was rapidly excised. In those that were killed at 15 minutes, 2 and 6 hours, 1 ml of black ink was injected intravenously immediately before sacrifice to delineate the ischemic region of the left ventricle (Fig. 1). No staining was necessary in rats killed 18 or 24 hours after occlusion because the infarct was evident at inspection as a thin, discolored and dyskinetic zone.

Quantification of proteolysis in nonischemic and ischemic ventricular myocardium. The ischemic region was rapidly excised from the rest of the heart, and the ischemic and nonischemic portions were immediately immersed in ice-cold "ischemic" or "aerobic" medium, respectively. The "aerobic" medium was a Krebs-Ringer bicarbonate buffer (23) equilibrated with a mixture of 95% oxygen (O_2) and 5% carbon dioxide (CO_2), pH 7.4, and supplemented with 10 m*M* glucose, 0.1 U/ml of insulin (Sigma Chemical Co.), 100 U/ml of penicillin and 100 μ/ml of streptomycin (Grand Island Biological Co.). This medium resembles the extracellular fluid in normally perfused tissues (24). The "ischemic" medium differed from the aerobic in that it had a lower bicarbonate content (13 versus 27 mEq/liter), it was saturated with a mixture of 90% nitrogen (N_2) and 10% CO_2 and, therefore, had a lower pH (6.8); furthermore, the solution lacked glucose and insulin. This medium resembles the extracellular fluid of ischemic tissues, in which the pressure of carbon dioxide (P_{CO_2}) increases, pH decreases to approximately 6.8 (25) and nutrients and hormones are lacking.

Subepicardial slices with an average weight of 70 mg were rapidly obtained from nonischemic and ischemic tissue using a chilled Stadie-Riggs microtome. Slices were gently blotted, divided into halves and weighed. One half of each slice was immediately homogenized in either the aerobic or ischemic medium (as described later) to determine tyrosine concentration. This value was assumed to represent the tyrosine concentration in the incubated tissue at the beginning of incubation. The other half of the slice was placed in a 25 ml sterile Erlenmeyer flask containing 3.0 ml of aerobic or ischemic medium. Both incubation media were supplemented with cycloheximide, 0.5 m*M* (Sigma Chemical Co.); this concentration has been shown to inhibit cardiac protein synthesis by more than 95% (20). The atmosphere above the aerobic or ischemic medium was equilibrated with 95% O_2 and 5% CO_2 or 90% N_2 and 10% CO_2, respectively, by gassing flasks with the appropriate gas mixture for 10 minutes. Slices were incubated for 1 hour at 37°C with continuous gentle shaking (approximately 80 cycles/min). Unless otherwise specified, nonischemic and ischemic tissues were incubated with aerobic and ischemic medium, respectively.

Determination of tyrosine release. At the end of the incubation period, each slice was removed from its medium. Protein was removed from a 2.0 ml sample of the incubation medium by trichloroacetic acid precipitation (final concentration, 10%) and centrifugation at 2,500 *g* for 30 minutes; a 2.0 ml aliquot of the supernatant was assayed for tyrosine (26) to determine the amount of tyrosine released into the medium during incubation. Each slice was blotted and immediately homogenized in 1.0 ml of fresh incubation medium; the homogenizing tubes were rinsed with an additional 1.0 ml of medium. Homogenate and wash were combined with 0.5 ml of ice-cold 50% trichloroacetic acid, mixed and centrifuged at 2,500 *g* for 30 minutes. A 2.0 ml aliquot of supernatant was removed and assayed for tyrosine to determine slice concentration of tyrosine at the end of incubation. In 789 samples, the protein pellets of both homogenate and medium were dissolved in 2.0 ml of 1 *M* sodium hydroxide. An aliquot of this solution was assayed for proteins by the method of Lowry et al. (27).

Tyrosine release was calculated by adding the amount released into the medium during incubation to the amount present in the slice at the end of incubation and then subtracting the tyrosine content of the slice at the beginning of incubation. Tyrosine release was related to tissue wet weight

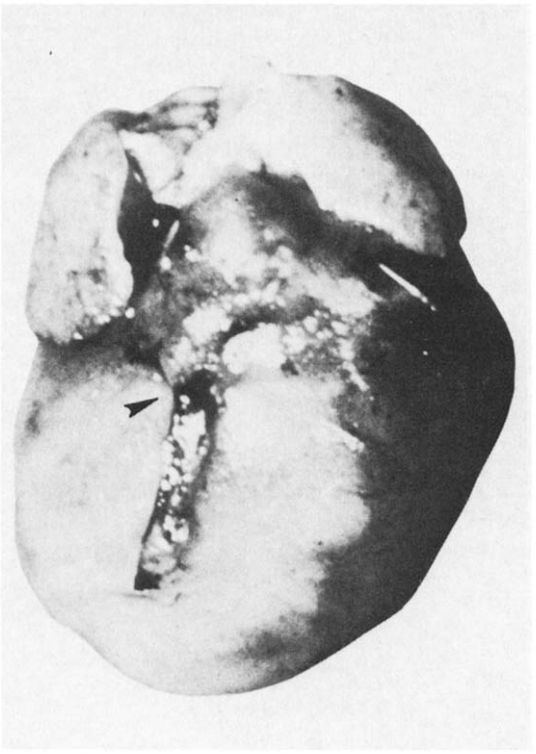

Figure 1. A typical example of demarcation of ischemic region by intravenous injection of black ink. The ischemic region is evident as a large unstained area (**white**) occupying most of the left ventricular free wall and clearly separated from the normally perfused myocardium (**black**). The **arrow** indicates the coronary ligature.

in all experiments and, in most experiments, also to the amount of protein available for breakdown in the slice; results obtained with the two methods were similar. Protein available for breakdown in the slice was computed as follows: the protein concentration in the homogenized half of each original slice was assumed to also represent the concentration in the incubated half at the beginning of incubation. This value was multiplied by the wet weight of the incubated tissue to give total protein content at the start of incubation. By subtracting from this quantity the amount of protein leaked into the medium during incubation (determined on the trichloroacetic acid precipitate from the medium sample), the total amount of protein available for degradation in the slice was calculated.

Linearity of tyrosine release during 1 hour of incubation was assessed in different tissues: nonischemic ventricular myocardium of rats not undergoing coronary occlusion and 15 minute and 6 hour ischemic ventricular myocardium. Slices of each tissue were incubated for 20, 40 or 60 minutes, and tyrosine release was measured as described.

Evaluation of the effects of antipain, leupeptin, pepstatin and chymostatin on proteolysis in nonischemic and ischemic ventricular myocardium. Multiple experiments were conducted to assess the effects of proteinase inhibitors, administered in vivo separately or in combination, on myocardial proteolysis. The details of these experiments are specified in Table 1. The water-soluble agents, antipain and leupeptin, were dissolved in normal saline solution, whereas pepstatin and chymostatin, which are minimally soluble in water, were dissolved in dimethylsulfoxide. In Groups 1 to 12, in which coronary artery occlusion was performed, proteolysis was measured in both ischemic and nonischemic myocardium. Determination of proteolysis was limited to nonischemic myocardium in Groups 13 to 18, which did not undergo coronary occlusion.

Proteolysis in atria. Tyrosine release by atrial myocardium was determined in rats used as controls for assessment of ventricular proteolysis (Group 13). The right and left atria were excised and blotted gently. Part of the atria was homogenized immediately and the remainder was incubated in aerobic medium. The rest of the experiment was conducted as described previously.

Proteolysis during the first 24 hours of acute myocardial infarction. To determine the changes in proteolysis that occur during the development of ischemic necrosis, tyrosine release was measured in slices of nonischemic or ischemic myocardium, or both, obtained from control rats not subjected to occlusion (Group 13) and from control rats at various times after coronary occlusion: 15 minutes (Group 5), 1 hour, 2 hours (Group 1), 6 hours (Group 8) and 18 and 24 hours. To assess the effect of the incubation medium itself on proteolysis, replicate slices of nonischemic and ischemic myocardium were obtained from control rats killed 2 hours after coronary occlusion (Group 1); one slice was incubated in aerobic medium and the other in ischemic medium.

Statistical analysis. All values are expressed as mean ± standard error of the mean. Differences between two groups were analyzed by unpaired Student's t test. One way analysis of variance was used for simultaneous multiple comparisons; if the F test revealed significant differences among groups, the level of significance for each comparison was determined by the Bonferroni method (28).

Results

The rate of tyrosine release remained approximately constant for 1 hour (incubation time used in this study) in both 15 minute and 6 hour ischemic myocardium as well as in nonischemic myocardium (Fig. 2). Stability of tyrosine release by tissue preparations during a 1 hour incubation afforded valid comparisons between control and treated rats.

Changes in myocardial proteolysis after coronary artery occlusion. Figure 3 illustrates proteolysis in control rats not subjected to coronary occlusion and in control rats sacrificed at various times after occlusion. Although proteolysis in nonischemic myocardium decreased somewhat

Table 1. Protocols of the Experiments With Proteinase Inhibitors

Group	Treatment	Vehicle	Route of Administration	Interval From Occlusion to Sacrifice
1	NS, 0.25 ml 10 min preocclusion	—	iv	2 h
2	A, 12 mg/kg 20 min plus 8 mg/kg 10 min preocclusion (total: 20 mg/kg)	NS, 0.25 ml	iv	2 h
3	L, 10 mg/kg 10 min preocclusion	NS, 0.25 ml	iv	2 h
4	L, 40 mg/kg 10 min preocclusion	NS, 0.25 ml	iv	2 h
5	NS, 0.3 ml 45 min plus 0.4 ml 30 min plus 0.2 ml 15 min plus 0.3 ml 1 min preocclusion (total: 1.2 ml)	—	iv	15 min
6	A, 12 mg/kg 45 min plus A, 8 mg/kg and L, 20 mg/kg 30 min plus L, 20 mg/kg 15 min preocclusion (total: A, 20 mg/kg plus L, 40 mg/kg)	NS as in Group 5	iv	15 min
7	A, 12 mg/kg and P, 1.3 mg/kg 45 min plus A, 8 mg/kg and L, 20 mg/kg and P, 1.7 mg/kg 30 min plus L, 20 mg/kg and P, 0.8 mg/kg 15 min plus P, 1.2 mg/kg 1 min preocclusion (total: A, 20 mg/kg plus L, 40 mg/kg plus P, 5 mg/kg)	NS as in Group 5	iv	15 min
8	NS as in Group 5 preocclusion plus 1.2 ml 2 h postocclusion plus 1.2 ml 4 h postocclusion	—	iv preocclusion ip postocclusion	6 h
9	A plus L plus P as in Group 7 preocclusion plus A, 20 mg/kg and L, 40 mg/kg and P, 5 mg/kg 2 h postocclusion plus A, 20 mg/kg and L, 40 mg/kg and P, 5 mg/kg 4 h postocclusion	NS as in Group 8	iv preocclusion ip postocclusion	6 h
10	DMSO, 1 ml 18 h plus 1 ml 1 h preocclusion	—	ip	3 h
11	P, 8 mg/kg 18 h plus 8 mg/kg 1 h preocclusion	DMSO, 2 ml	ip	3 h
12	C, 60 mg/kg 18 h plus 60 mg/kg 1 h preocclusion	DMSO, 2 ml	ip	3 h
13	NS, 0.4 ml 20 min presacrifice	—	iv	No occlusion
14	A, 20 mg/kg 20 min presacrifice	NS, 0.25 ml	iv	No occlusion
15	L, 40 mg/kg 20 min presacrifice	NS, 0.25 ml	iv	No occlusion
16	NS, 1.0 ml 18 h plus 1.0 ml 1 h presacrifice	—	ip	No occlusion
17	DMSO, 1.0 ml 18 h plus 1.0 ml 1 h presacrifice	—	ip	No occlusion
18	P, 8 mg/kg 18 h plus 8 mg/kg 1 h presacrifice	DMSO, 2 ml	ip	No occlusion

A = antipain; C = chymostatin; DMSO = dimethylsulfoxide; i.p. = intraperitoneally; i.v. = intravenously; L = leupeptin; NS = normal saline solution; P = pepstatin. Braces identify different experiments.

after coronary occlusion (possibly as a result of surgical trauma or hemodynamic changes), differences were not statistically significant. Proteolysis in ischemic myocardium was similar at 15 minutes and at 1 and 2 hours after coronary occlusion, but decreased significantly by 6 hours (probability [p] < 0.01 versus 15 minutes). Proteolysis then increased, reaching values at 18 hours after occlusion similar to those observed at 2 hours. At 24 hours after occlusion, proteolysis was significantly ($p < 0.001$) greater than at 6 hours, but not statistically different from that measured during the first 2 hours. At no time during the first 24 hours of occlusion was proteolysis significantly greater in ischemic than in nonischemic myocardium. These results cannot be attributed to the lack of insulin in the ischemic medium because the hormone reduces cellular protein degradation (29).

We also evaluated the effect of the medium itself on proteolysis in control tissues obtained 2 hours after coronary occlusion (Group 1, Fig. 4). Nonischemic myocardium exhibited a lower ($p < 0.05$) rate of proteolysis in ischemic than in aerobic medium, possibly as a result of hypoxia and acidosis, or two conditions associated with depression in intracellular protein degradation (18). In contrast, proteolysis in 2 hour ischemic myocardium did not differ signifi-

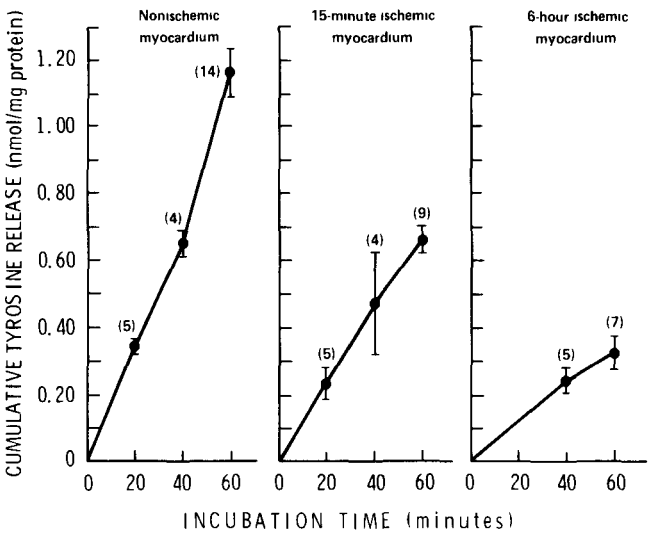

Figure 2. Cumulative tyrosine release from slices as a function of incubation time. **Numbers in parentheses** indicate the number of rats studied at each time point. **Vertical bars** indicate 1 standard error of the mean.

cantly in the two media, perhaps reflecting the fact that severe ischemia-induced damage had rendered the proteolytic machinery insensitive to variations of the environment. Moreover, we compared nonischemic and ischemic tissues incubated in the same media, thus eliminating differences due to the medium itself. Figure 4 demonstrates that proteolysis was not greater in ischemic than in nonischemic myocardium even when the two tissues were incubated with the same medium (either aerobic or ischemic).

Effects of antipain, leupeptin, pepstatin and chymostatin on proteolysis in ischemic myocardium. The effects of antipain (20 mg/kg), leupeptin in "low" dose (10 mg/kg) and leupeptin in "high" dose (40 mg/kg), administered 10 minutes before coronary occlusion, on proteolysis in ischemic myocardium 2 hours after occlusion are illustrated in Figure 5. Antipain reduced the rate of protein breakdown by 60% ($p < 0.001$). Leupeptin inhibited proteolysis in a dose-related fashion (−49% in low dose and −72% in high dose, $p < 0.001$).

The effects of pepstatin and chymostatin on proteolysis in ischemic myocardium are presented in Table 2. Treatments were started 18 hours before sacrifice to allow maximal absorption of the inhibitors (21). Proteolysis in 3 hour ischemic myocardium was not significantly different among rats pretreated with pepstatin (16 mg/kg), chymostatin (120 mg/kg) or dimethylsulfoxide (8 ml/kg). These results may be due to a relative inability of pepstatin and chymostatin to cross cell membranes (30–32) or to an inhibitory effect of dimethylsulfoxide itself on myocardial proteolysis, or both (see later).

The effects of combinations of antipain, leupeptin and pepstatin, administered before coronary occlusion, on proteolysis in ischemic myocardium are illustrated in Figure 5. Proteolysis in 15 minute ischemic myocardium was markedly decreased (−65%, $p < 0.001$) by simultaneous pretreatment with antipain (20 mg/kg) and leupeptin (40 mg/kg) and almost completely suppressed (−88%, $p < 0.001$) by the combination of antipain (20 mg/kg), leupeptin (40 mg/kg) and pepstatin (5 mg/kg). The latter combination readministered 2 and 4 hours after coronary artery ligation caused a prolonged inhibition of proteolysis, as demonstrated by the effect observed in 6 hour ischemic myocardium (−72%, $p < 0.05$).

Effects of antipain, leupeptin, pepstatin and chymostatin on proteolysis in nonischemic myocardium. The effects of antipain and leupeptin on protein degradation in nonischemic myocardium are summarized in Table 3. A tendency toward decrease in proteolysis (−29%) was observed in rats treated with antipain, but it did not achieve statistical significance ($p = 0.06$). In contrast, leupeptin in high dose markedly inhibited proteolysis (−54%, $p < 0.02$). The combination of antipain, leupeptin and pepstatin decreased proteolysis by 43% ($p < 0.05$). This decrease was not significantly different from that produced by leupeptin alone.

The influence of pepstatin and chymostatin on protein degradation in nonischemic myocardium is presented in Table 2. When aerobic medium was used, no significant dif-

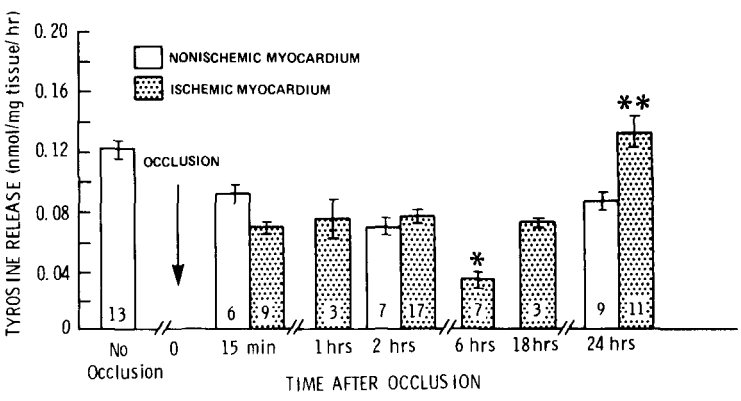

Figure 3. Tyrosine release from slices of nonischemic (**white bars**) or ischemic (**dotted bars**) myocardium incubated in aerobic or ischemic medium, respectively. Results are mean ± standard error of the mean. **Numbers at the bottom of each bar** indicate the number of rats studied. *$p < 0.01$ versus ischemic myocardium 15 minutes after coronary occlusion; **$p < 0.001$ versus ischemic myocardium 6 hours after coronary occlusion.

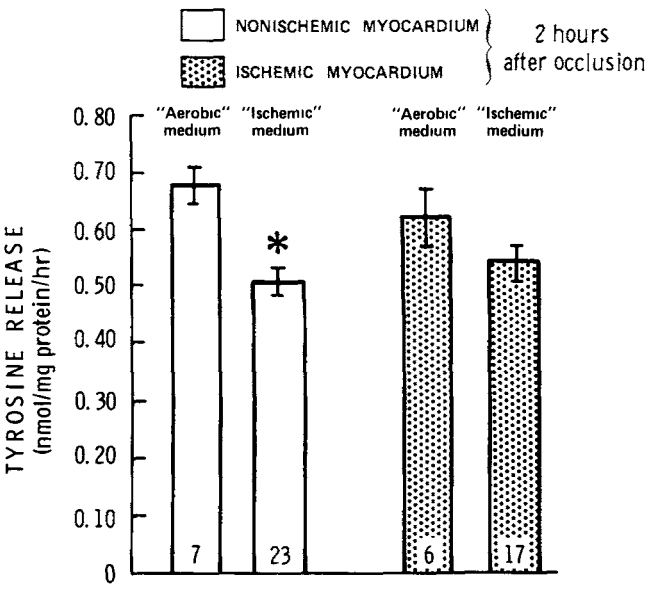

Figure 4. Effect of incubation medium on tyrosine release from slices of nonischemic (**white bars**) and ischemic (**dotted bars**) myocardium obtained 2 hours after coronary artery occlusion. Results are mean ± standard error of the mean. **Numbers at the bottom of each bar** indicate the number of rats studied. *p < 0.05 versus nonischemic myocardium in aerobic medium.

ference was observed between rats receiving pepstatin (16 mg/kg, Group 18) and those receiving the vehicle dimethylsulfoxide (Group 17); however, dimethylsulfoxide itself effected a marked inhibition of proteolysis (−61%, $p < 0.01$ compared with values in the rats injected with saline solution, Group 16). It is possible that this effect of the vehicle might have masked an action of pepstatin on myocardial proteolysis. When ischemic medium was used, proteolysis tended to be lower in pepstatin-treated rats (Group 11) than in control rats receiving dimethylsulfoxide (Group 10); however, the difference did not achieve statistical significance. Chymostatin (120 mg/kg) did not produce any appreciable effect on proteolysis.

Proteolysis in atria. Tyrosine release by atrial preparations incubated in aerobic medium was 1.57 ± 0.18 nmol/mg protein per hour; release of tyrosine into the medium was 0.15 ± 0.01 nmol/mg tissue per hour.

Discussion

Modeling considerations. This investigation provides a new model for studies of proteolysis during myocardial infarction. We measured the rate of protein breakdown in nonischemic and ischemic rat myocardium by incubating tissue slices in media supplemented with cycloheximide to block protein synthesis (20) and by quantifying tissue release of tyrosine. Because this amino acid is neither synthesized nor metabolized by cardiac muscle (20), its release in the presence of cycloheximide reflects the degradation of tissue proteins to amino acids (20,24). Most studies of cardiac protein turnover during ischemia have used Langendorff preparations (17,18). Our model offers several advantages over such preparations: it affords quantification of proteolysis in regional acute myocardial ischemia produced in vivo, and in specific regions (center of ischemic zone, nonischemic zone); furthermore, proteolysis can be assessed in these regions even after the first few hours of coronary occlusion. This model, therefore, may be useful for investigations of regional myocardial metabolism during acute infarction, especially if serial measurements over a prolonged period of time are needed.

The rationale for the selection of the incubation medium was to determine the rate of proteolysis in each tissue under conditions similar to those present in vivo. We therefore incubated ischemic myocardium in ischemic medium (which resembles extracellular fluid in conditions of acute flow deprivation) and nonischemic myocardium in aerobic medium (which resembles extracellular fluid of normally perfused tissues).

The validity of our measurements of proteolysis is confirmed by the data from atrial preparations. Tyrosine release

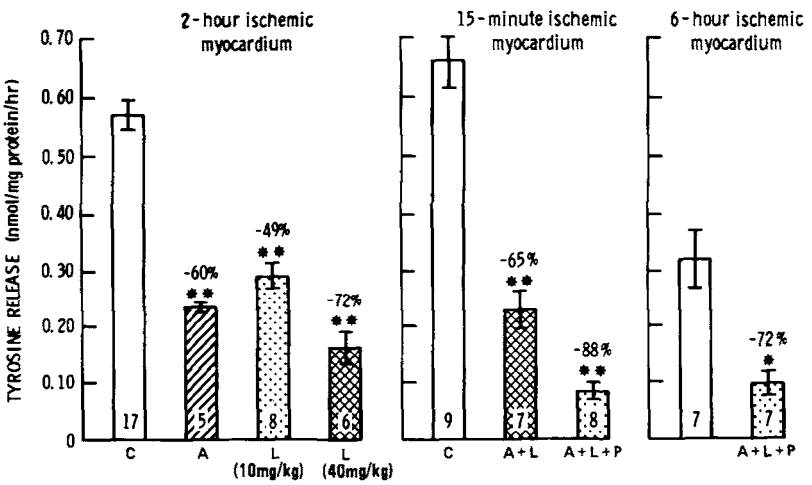

Figure 5. Effect of in vivo pretreatment with protease inhibitors on proteolysis in ischemic myocardium. Results are mean ± standard error of the mean. **Numbers at the bottom of each bar** indicate the number of rats studied. A = antipain; C = control; L = leupeptin; P = pepstatin; *p < 0.05, **p < 0.001 versus control group.

Table 2. Effects of Dimethylsulfoxide, Pepstatin and Chymostatin on Proteolysis in Ischemic and Nonischemic Myocardium

Intervention	Tyrosine Release (nm/mg protein per h)	Change From Control
	A. 3 Hour Ischemic Myocardium	
Control (DMSO, 8 ml/kg)	0.25 ± 0.05 (n = 7)	
DMSO, 8 ml/kg plus pepstatin, 16 mg/kg	0.20 ± 0 06 (n = 6)	−20%
DMSO, 8 ml/kg plus chymostatin, 120 mg/kg	0 23 ± 0.04 (n = 6)	−8%
	B Nonischemic Myocardium	
	No occlusion, aerobic medium	
Control (saline solution, 8 ml/kg)	1.16 ± 0 18 (n = 9)	
DMSO, 8 ml/kg	0.45 ± 0 08* (n = 7)	−61%
DMSO, 8 ml/kg plus pepstatin, 16 mg/kg	0.43 ± 0.06 (n = 7)	−63%
	3 Hours after occlusion, ischemic medium	
Control (DMSO, 8 ml/kg)	0 29 ± 0.06 (n = 12)	
DMSO, 8 ml/kg plus pepstatin, 16 mg/kg	0.13 ± 0.02 (n = 5)	−55%
DMSO, 8 ml/kg plus chymostatin, 120 mg/kg	0 25 ± 0.04 (n = 3)	−14%

*p < 0.01 versus saline solution; values are mean ± standard error of the mean; DMSO = dimethylsulfoxide

by such preparations (1.57 ± 0.18 nmol/mg protein per hour) was virtually identical to that reported by Curfman et al. (20) (1.59 ± 0.16 nmol/mg protein per hour) in similar conditions of incubation. Furthermore, release of tyrosine into the medium (0.15 ± 0.01 nmol/mg tissue per hour) was similar to that reported by Libby and Goldberg (30) (0.33 ± 0.02 nmol/mg tissue per 2 hours).

Tissue slices were obtained with a Stadie-Riggs microtome; minimal pressure was applied to the tissue to reduce damage. Preliminary experiments showed that all slices obtained with this technique were approximately 0.5 mm thick, which allows diffusion of gases and soluble molecules to and from the center of the section (33). To minimize tissue damage, sections were made parallel to the epicardial surface (that is, parallel to the orientation of the myocardial fiber bands) and subepicardial slices were used. It has been shown previously (34) that although the outermost layers are damaged by mechanical trauma, most cells in sections obtained by gentle slicing maintain near normal ultrastructural appearance as well as volume and ionic composition of intracellular compartment.

In the present experiment, slices of nonischemic ventricle incubated in aerobic medium (which contains insulin) released tyrosine at a rate of 0.122 ± 0.015 nmol/mg tissue per hour. This value is reasonably close to that reported by McKee et al. (35) (0.196 ± 0.010 nmol/mg tissue per hour) in intact rat hearts perfused with insulin-free medium. The difference between the two values may be accounted for by the inhibition exerted by insulin on myocardial proteolysis (29). The lower rates of proteolysis in our model may also be due to the inability of the damaged outer layers of the slice to maintain the high energy phosphate levels necessary for intracellular proteolysis (36). However, this possibility would lead to underestimation of the rate of proteolysis in nonischemic myocardium but not in ischemic, energy-depleted tissue. Because our results indicate that protein breakdown in ischemic myocardium is not greater than in nonischemic myocardium, underestimation of proteolysis in the latter would strengthen our conclusions.

Proteolysis during the evolution of myocardial infarction. If either lysosomal or cytoplasmic proteinases were activated early in the course of myocardial ischemia in sufficient quantity to precipitate substantial irreversible damage, this increased enzyme activity might be manifested as a generalized increase of myocardial proteolysis soon after occlusion. Our data indicate that overall proteolysis does not increase throughout the phase of acute myocardial ischemia during which irreversible cell damage develops (Fig. 3). Thus, neither tissues studied very early in the development of ischemic damage (15 minute ischemic myocardium) nor those in which a more severe ischemic injury had occurred (2 hour ischemic myocardium) exhibited higher rates of proteolysis than nonischemic myocardium at corresponding times after occlusion. By 6 hours of occlusion,

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Table 3. Effects of Antipain, Leupeptin and Pepstatin on Proteolysis in Nonischemic Myocardium

Intervention	Tyrosine Release (nmol/mg protein per h)	Change From Control
	A. No Occlusion	
Control I (saline solution, 0.25 ml)	1.16 ± 0.14 (n = 14)	
Antipain, 20 mg/kg	0 82 ± 0.08† (n = 8)†	−29%
Leupeptin, 40 mg/kg	0.53 ± 0 07* (n = 5)	−54%
	B. 15 Min After Occlusion	
Control II (saline solution, 1.2 ml)	0.93 ± 0.14 (n = 6)	
Antipain, 20 mg/kg plus leupeptin, 40 mg/kg plus pepstatin, 5 mg/kg	0.53 ± 0.11 ‡(n = 9)	−43%

* $p < 0.001$ versus control I; † $p = 0.06$ versus control I; ‡ $p < 0.05$ versus control II. Values are mean ± standard error of the mean.

protein breakdown in ischemic myocardium actually decreased. At this time, irreversible damage has occurred in almost all of the ischemic region (19). Even if it is assumed that further damage occurs after 6 hours, our data obtained at 18 and 24 hours indicate that such damage is not mediated by an increase in cellular proteinase activity. No evidence of increased proteolytic activity in ischemic tissues was were incubated in the same medium (Fig. 4). These results are consistent with those obtained previously in this laboratory in a canine model of acute myocardial ischemia (37). Failure of proteolysis to increase despite release of lysosomal cathepsins most likely results from the lack of energy phosphates associated with ischemia. Indeed, an energy requirement for intracellular proteolysis has been documented in a variety of models (16,18,36).

Our study represents the first assessment of myocardial proteolysis throughout the phase of acute myocardial infarction during which irreversible injury develops. Our findings indicate that lysosomal and, more generally, cellular proteinases do not undergo a generalized activation during this phase, suggesting that proteolytic enzymes do not contribute importantly to ischemic myocellular death. However, failure of overall proteolysis to increase during acute myocardial infarction does not disprove the hypothesis that ischemia might selectively accelerate breakdown of vital cellular proteins while reducing degradation of other components less crucial for immediate cell survival (16). According to this hypothesis, unchanged levels of overall proteolysis during ischemia may reflect the net balance between activation of key enzymes causing cell damage and depression of other proteinases. Hence, without additional evidence, the possibility that even baseline levels of proteolysis may contribute to ischemic injury cannot be definitively refuted.

Effects of proteinase inhibitors in ischemic myocardium. This hypothesis could be tested by suppressing proteolysis in acutely ischemic myocardium and by evaluating the effect of such suppression on the extent of ischemic damage. Therefore, in an attempt to determine whether such an approach was feasible in the design of subsequent investigations, we studied the potential of proteinase inhibitors to inhibit proteolysis in our model of acute myocardial infarction.

Both leupeptin and antipain, administered in vivo before coronary occlusion, produced a marked inhibition of proteolysis in ischemic myocardium in the early phase of myocardial infarction (Fig. 5). The combination of antipain and leupeptin did not reduce protein breakdown any more than did antipain alone (Fig. 5), suggesting that antipain exerted its action by inhibiting leupeptin-sensitive enzymes (cathepsin B, calcium-activated neutral protease). Addition of pepstatin to the combination of antipain and leupeptin resulted in greater inhibition of proteolysis (−88 versus −65%). Although the difference was not statistically significant, it suggests an effect of pepstatin on protein breakdown of ischemic tissues. The combination of antipain, leupeptin and pepstatin readministered 2 and 4 hours after coronary occlusion inhibited proteolysis even at 6 hours of ischemia (Fig. 5). Thus, an almost complete suppression of protein breakdown was produced by this combination in the ischemic region throughout the phase of myocardial infarction during which irreversible damage develops.

These results represent the first successful attempt to interfere with proteolysis in ischemic myocardium in vivo, and have two important implications. First, they elucidate the mechanisms of protein degradation during acute myocardial infarction. Our data indicate that this process is largely mediated by the enzymes sensitive to antipain, leupeptin and pepstatin: lysosomal cathepsins A, B, D, L and H and cytoplasmic calcium-activated neutral protease. Second, these proteinase inhibitors appear to be a potentially useful tool for assessing the significance of proteolysis in the genesis of ischemic myocellular death.

Effects of proteinase inhibitors in nonischemic myocardium. Unlike its effects in ischemic myocardium, antipain did not decrease tyrosine release significantly in non-

ischemic myocardium (Table 3). This difference may depend on a greater permeability of the cell membrane of ischemic myocytes to the inhibitor. Alternatively, the activity of antipain-sensitive enzymes (cathepsins A and B and calcium-activated neutral protease) might be enhanced during ischemia. A small decrease in total proteolysis (−19%) has been reported in cultured fetal mouse hearts exposed to antipain (30 μM) for 2 days (31). Because proteolysis was 29% lower in antipain-treated than in control rats in the present study and because this difference almost reached statistical significance ($p = 0.06$), our data are not inconsistent with a minor effect of antipain on protein breakdown in nonischemic myocardium.

Leupeptin (40 mg/kg) markedly inhibited proteolysis (Table 3), an effect similar to that observed in ischemic myocardium. These data provide the first demonstration of an inhibition of myocardial proteolysis by in vivo administration of leupeptin, and confirm results obtained by other investigators in different models (31,38). Because leupeptin inhibits thiol-proteases (cathepsins B, L and H and calcium-activated neutral protease [21,22]), our results identify a major role of this class of enzymes in normal cardiac protein turnover.

Unlike the results found in ischemic tissues, the combination of antipain, leupeptin and pepstatin did not inhibit proteolysis in nonischemic myocardium any more than did leupeptin alone (Table 3). These data suggest that antipain and leupeptin do not have additive effects in normal myocardium and pepstatin, administered in vivo, does not interfere with protein breakdown in this tissue. The latter inference is substantiated by the failure of pepstatin (administered starting 18 hours before sacrifice to allow maximal absorption [21]) to inhibit proteolysis in nonischemic myocardium (Table 2). This result, which is consistent with studies in other models (30,31,38), may reflect the relative impermeability of normal sarcolemma to pepstatin (30–32). Incubation of nonischemic myocardium in ischemic medium, by reducing cell membrane integrity, might facilitate entry of pepstatin into myocytes. We therefore administered pepstatin starting 18 hours before sacrifice and measured tyrosine release from slices of nonischemic ventricle incubated in ischemic medium (Table 2). Proteolysis was 55% lower in pepstatin-treated than in control rats. The difference did not achieve statistical significance due to the scatter of the data; nevertheless, this result suggests that an effect of pepstatin on myocardial proteolysis may become manifest in conditions of impaired cell membrane integrity.

Chymostatin did not affect proteolysis in nonischemic myocardium (Table 2), possibly because of the poor permeability of normal cell membranes to this compound (31).

We are grateful to Mary Slegowski for her technical assistance and to Paula Parisius for performing protein determinations

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